

## Early Detection of a Two-Long-Terminal-Repeat Junction Molecule in the Cytoplasm of Recombinant Murine Leukemia Virus-Infected Cells

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**We showed that a U5-U3 junction was reproducibly detected by a PCR assay as early as 1 to 2 h postinfection with a DNase-treated murine leukemia virus (MLV)-containing supernatant in aphidicolin-arrested NIH 3T3 cells, as well as in nonarrested cells. Such detection is azidothymidine sensitive and corresponded to neosynthesized products of the reverse transcriptase. This observation was confirmed in two additional human cell lines, TE671 and ARPE-19. Using cell fractionation combined with careful controls, we found that a two-long-terminal-repeat (two-LTR) junction molecule was detectable in the cytoplasm as early as 2 h post virus entry. Altogether, our data indicated that the neosynthesized retroviral DNA led to the early formation of structures including true two-LTR junctions in the cytoplasm of MLV-infected cells. Thus, the classical assumption that two-LTR circles are a mitosis-dependent dead-end product accumulating in the nucleus must be reconsidered. MLV-derived products containing a two-LTR junction can no longer be used as an exclusive surrogate for the preintegration complex nuclear translocation event.**

Following retrovirus entry into the cytoplasm, the RNA genome is reverse transcribed to a double-stranded linear DNA with two long terminal repeat (LTR) sequences. Reverse transcription (RT) occurs in an intracellular complex made of cellular and viral proteins termed the preintegration complex (PIC) (2). During and after mitosis, the PIC penetrates the nuclear compartment and the viral DNA integrates, as recently shown, into the human host cell genome near the start of transcriptional units (48). This event is mediated by the viral integrase (IN). Viral integration results in the elimination of 2 bp from each end of both LTRs (4, 5, 17). However, sequence analysis of the host cell genome-provirus junctions was somewhat heterogeneous. Linear DNA was described as the precursor of integration since the murine leukemia virus (MLV) PIC isolated from the cytoplasm of infected cells and containing the linear forms of viral DNA was competent to carry out integration *in vitro* (2, 4).

In addition to linear DNA, other forms of viral DNA were described, and at least two circular forms containing one and two LTRs resulting from the linear DNA circularization were found in the nucleus (41, 42). One- and two-LTR circles are believed to result from homologous recombination between the LTRs or from direct end-to-end joining of the linear DNA, respectively (41). These circular forms are often defined as “dead-end” products because they were described as ineligible for integration (5). Additionally, they are generally used as an index for nuclear import of the PIC from either retroviruses or lentiviruses (28, 38). As a result, many restrictions in retroviral

or lentiviral replication associated with the absence of one- and/or two-LTR circles were interpreted as blockage of PIC nuclear import (26, 32, 39, 50, 51).

As opposed to lentiviruses, nuclear import of the MLV PIC and the consequent viral integration require passage through mitosis of the target cells (28, 38). The mechanism by which the MLV PIC enters the nucleus remains unclear, and the requirement for mitosis for MLV but not for human immunodeficiency virus (HIV) nuclear import was supposed to be simply due to the large size of the MLV nucleoprotein complex (2, 16). Nevertheless, neither the MLV nor the HIV PIC enters the nucleus by passive transport. Indeed, nuclear import of HIV was reported to be an active and energy-dependent process (6). Furthermore, the MA, Vpr, and IN proteins of HIV have been reported to carry the nuclear localization signal and have long been considered to play a role in nuclear targeting (7, 18, 21). However, HIV type 1 (HIV-1) replication in non-dividing cells can occur even in the absence of the Vpr or MA protein (37). Other factors contributing to the nuclear import of HIV, such as the presence of the central DNA flap (PPTc) and a valine residue at position 165 in the nuclear localization signal of IN, were recently reported (1, 53). However, the contributions of these factors are still controversial (14, 29).

The present study was initiated by an unexpected observation. Indeed, we previously reported a producer cell line-dependent restriction of an amphotropic MLV replication characterized by defective nuclear import of the reverse-transcribed viral DNA (40). To further analyze this restriction, we looked for early viral DNA forms in the cytoplasm of replication-defective recombinant MLV-infected cells. Since nondividing cells cannot support nuclear translocation of the PIC from MLV, and consequently two-LTR circle formation (28, 38), we used aphidicolin-arrested MLV-infected cells as a

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control. However, and surprisingly, two-LTR junctions were reproducibly detected by PCR in aphidicolin-arrested MLV-infected cells. Using a replication-defective and a replication-competent MLV vector, we reexamined the viral DNA products neosynthesized early following cell entry and the mitosis requirement for the generation of the two-LTR junctions. Previous studies established that viral circular DNA molecules appeared only if integration occurred and vice versa. Therefore, their appearance has been used as a marker for entry of viral DNA into the nucleus since these circular DNA molecules are believed to be formed only in the nucleus (4, 38). Other studies of MLV infection of aphidicolin-arrested or proliferative cells found that both integration and circularization of linear DNA occurred late in the cell cycle and required mitosis (27, 28, 38). Indeed, infecting G<sub>1</sub>/S-arrested cells with MLV resulted in the detection of two-LTR circle junctions 8 h after the cells were released and underwent a complete cycle (28). Recently, a comparison between retrovirus vectors demonstrated that aphidicolin-arrested cells could be infected by avian sarcoma virus but not by MLV vectors (20, 25). In these studies, circular viral DNA molecules were looked for 16 to 24 h post viral infection as an indicator of PIC nuclear translocation and productive infection.

The present study provides evidence that two-LTR junction molecules are generated in both replication-defective and replication-competent MLV-infected cells soon after the completion of RT and that their generation does not require target cell mitosis. Additionally, MLV vector-mediated transduction of aphidicolin-arrested cells remains ineffective, indicating that the detection of a two-LTR junction sequence, particularly at early time points, is not an exclusive indicator for PIC nuclear translocation.

#### MATERIALS AND METHODS

**Cells and retroviral infection protocol.** A549 cells (ATCC CCL185) were derived from a human bronchocarcinoma, HT1080 cells (ATCC CCL2) were derived from a human fibrosarcoma, TE671 cells (ATCC CRL8805) were derived from a human medulloblastoma, NIH 3T3 cells (ATCC CRL1658) were from a murine embryo fibroblast line, and ARPE-19 cells were from a human retinal pigmented epithelial cell line (13). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum.

Replication-competent MLV strain RRV-IRES was described previously (45). Briefly, it was made of the entire genome of Moloney MLV in which the Moloney MLV envelope was replaced with the amphotropic 4070A envelope and to which an internal ribosomal entry site (IRES), followed by the enhanced green fluorescence protein cDNA, was introduced downstream of the *env* sequence. RRV-IRES production relied on transfection and subsequent propagation in NIH 3T3 cells. The replication-defective recombinant MLV vector containing the MFGnlsLacZ recombinant genome was stably produced from the TA7 producer cell line (gift from F. L. Cosset) (10). Viral titration for both replication-competent and -defective viruses used TE671 indicator cells as described in reference 12 and routinely found at 10<sup>5</sup> to 10<sup>6</sup> and 10<sup>6</sup> to 10<sup>7</sup> transducing U/ml, respectively. Prior to cell transduction, viral supernatants were first treated with Benzonase (Merck) for 1 h at room temperature to remove extracellular viral DNA from cell debris. Cell lines were then infected with TA7-derived vector or RRV-IRES at multiplicities of infection (MOI) of 1 and 0.05 to 0.5, respectively, in the presence of Polybrene (8-μg/ml final concentration). Production of recombinant MLV-derived retroviruses from A549 (A549-MLV) and HT1080 (HT1080-MLV) cells used the MFGnlsLacZ retroviral vector and recombinant adenoviruses encoding Gag-Pol, the 4070A Env, or the vesicular stomatitis virus G protein as described in references 12 and 40. Titers were determined as described above.

**Plasmids.** The pMFGnlsLacZ plasmid encoded a retroviral vector carrying the β-galactosidase reporter gene under the control of the LTR promoter (23). pPCR2.1U5/U3 was obtained by cloning into plasmid PCR2.1 (Invitrogen) the

U5-U3 junction amplified by PCR on total DNA extracted from MLV vector-infected TE671 cells 4 h postinfection. The U5 and U3 primers are described below.

**Detection of reverse-transcribed molecules.** Cells seeded in six-well plates (10<sup>6</sup>/well) were infected with Benzonase-treated TA7 supernatant for 2 h, washed twice in DMEM, and either recovered immediately or maintained in culture for 1 to 24 h. Control cells were preincubated with 50 μM azidothymidine (AZT, Retrovir) for 2 h renewed every 2 h. Total DNA was extracted, after cell trypsinization, with a QIAamp DNA kit (QIAGEN), and 0.7 μg of each sample was subjected to 35 cycles of PCR. The following primers were used for amplification of the different reverse-transcribed molecules (nucleotide positions correspond to those of the Moloney MLV sequence—GenBank accession number J02255): for LTRU3-*gag* DNA, the 5' primer (5'-GGCTCAGGGCCAAGAAGAGATGG) was located at nucleotides (nt) 7985 to 8007 and the 3' primer (5'-TTTTGGACTCAGGTCTGGGCTCT) was located at nt 393 to 414, which amplified a 692-bp fragment. This pair of primers amplified late RT products, synthesized after the second template switch. For the amplification of the two-LTR junction, one set of primers was used to obtain the U5-U3 molecules. The 5' primer (5'-ATCCGACTGTGGTCTCGCTG) was located at nt 68 to 87, and the 3' primer (5'-GAGTGAGGGGTTGTGGGCTCT) was located at nt 8241 to 8261, which amplified a 529-bp fragment. A control reaction was performed to detect the cytochrome *b* sequence by using two primers (5'-CCCCTCAGAAATGATATTGTCTCTCA-3' and 5'-CCATCCACATCTCAGCATGATGAAA), which amplified a 350-bp fragment. PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. When indicated, PCR products from an agarose gel were transferred onto Hybond plus nitrocellulose membrane (APB) and Southern blotted with fluorescein-labeled probes (Gene Images random prime labeling module; Amersham) specific for the full-length MLV DNA or the U5-U3 two-LTR junction fragment. On the following day, the membrane was processed in accordance with the manufacturer's protocol (Gene Images CDP-star detection module; Amersham) and expose to autoradiography film.

**Cell fractionation.** TE671 target cells (5 × 10<sup>6</sup>) were infected with MLV vectors and recovered by trypsinization at different times postinfection. Cells were washed with DMEM with no fetal calf serum. From this step, they were kept on ice and washed with 10 ml of hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 2 mM dithiothreitol). After centrifugation of the cells for 5 min at 400 × *g*, the cell pellet was resuspended in 400 μl of hypotonic buffer and 0.025% digitonin. The resuspended pellet was incubated for 10 min on ice and then centrifuged twice for 3 min at 1,000 × *g*. The supernatant corresponded to the cytoplasmic fraction. Before DNA extraction, the supernatant was diluted 1:2 in 2× proteinase K buffer (Tris at 20 mM [pH 8.0], EDTA at 25 mM, sodium dodecyl sulfate at 1%), after which 20 μl of proteinase K (10 mg/ml) was added and the mixture was incubated for 1 h at 37°C. Total DNA from the cytoplasm was then extracted by phenol-chloroform and ethanol precipitation.

To assay for contamination of cytoplasmic extracts by nuclear DNA, the cytoplasmic DNA fraction was subjected to β-globin PCR amplification with the 5' primer 5'-ACACAACTGTGTCTCACTAGC and the 3' primer 5'-CAACTTCATCCAGCTTCACC, which amplified a 110-bp fragment. To increase the readout sensitivity, samples from PCRs were then analyzed by Southern blotting with a β-globin-specific probe.

To test the absence of nuclear episomal DNA molecules in the cytoplasmic extract after cell fractionation, we used a G418-grown human kidney carcinoma cell line transfected with a plasmid containing the Epstein-Barr virus *ori*, EBNA, and neomycin genes under the control of the EBNA and simian virus 40 promoters, respectively (gift from Valérie Trichet-Migné, INSERM U463). The above-described cell fractionation procedure was applied to the cell line, and cytoplasmic and nuclear fractions were subjected to EBNA PCR amplification with the 5' primer 5'-TCCTGCTCCTGCTCCTGTTC and the 3' primer 5'-GC CGACCCAGCTTTTCTTC, which amplified a 655-bp fragment. PCR products were analyzed by Southern blotting with a 526-bp EBNA fragment (BspEI/EcoNI digest).

**Cell cycle analysis.** For cell cycle arrest at the G<sub>1</sub>/S border, NIH 3T3 cells were synchronized by serum starvation for 60 to 72 h and then treated with aphidicolin (Sigma) at 10 μg/ml for 18 h. To determine their cell cycle status, we stained cells with propidium iodide and subsequently analyzed their DNA contents by fluorescence-activated cell sorting.

**Quantitative PCR.** The primers (TIB MOLBIOL) that span the LTR and *gag* regions were 5' primer 5'-TGTCTGTCCGATTGTCTAGTGTCTA-3' (Moloney MLV sequence; GenBank accession number J02255) and 3' primer 5'-GGGTCCGCCAGATACAGAG-3'. The fluorogenic TaqMan probe used to detect the amplification products was MV-TM (5'-6FAM-TTAGCTAACTAGTACMGACGCAGGCGCA XT p-3'). The sequence corresponding to the two-LTR junction was amplified with 5' primer MV-LTRF (5'-GGAGGGTCTCTCTG

AGTGAT-3') and 3' primer MV-LTRA (5'-CTCAGTTATGTATTTTCCAT GCCTT-3'). The resulting amplicon was detected with the fluorogenic probe MV-LTR (5'-6FAM-AAAATGGCGTTACTTAAGCTAGCTTGCCAAXT p-3').

Reactions were done with 1× Light Cycler-FastStart DNA Master Hybridization Probes (Roche), each primer at 300 nM, 200 nM probe, and 5 µl of template DNA in a 20-µl volume. After the initial incubation at 95°C for 8 min, 45 cycles were carried out for 5 s at 95°C, followed by 8 s at 62°C. For each experiment, a standard curve of the amplicon being measured was run in duplicate ranging from 10 to 10<sup>5</sup> copies plus a no-template control. The pMFG-LZ plasmid was used for the standard curve of the LTR<sub>gag</sub> region, and pPCR2.1 containing the MLV two-LTR junction was constructed to establish the two-LTR reference curve.

Quantitation of the human β-globin- or murine glyceraldehyde-3-phosphate dehydrogenase-encoding gene, performed with the LightCycler-Control Kit DNA, was used to determine the number of cells. Reactions were analyzed with the LightCycler Software, version 3.5. Data are expressed as viral DNA copy numbers per million cells.

## RESULTS

**Passage through mitosis is not required for the generation of two-LTR junction molecules.** MLV infection requires target cell mitotic division (27, 33, 38), and the resulting two-LTR circular products are generally considered an index of the nuclear import of the PIC. However, while we were investigating a previously described MLV restriction phenotype (40), we reproducibly found two-LTR junction molecules by PCR in aphidicolin-arrested MLV-infected cells. Therefore, two-LTR junction molecules were specifically analyzed at early and late time points after virus entry in aphidicolin-arrested cells. NIH 3T3 cells were synchronized by serum starvation for 60 to 72 h and then arrested at the G<sub>1</sub>/S border after incubation with aphidicolin (10 µg/ml) for 18 h. Cells were then infected with replication-defective MLV vectors for different incubation periods (1, 2, 4, 8, and 24 h) at an MOI of 1. Subsequently, two-LTR junction and full-length viral DNA products (47), i.e., LTRU3-*gag* molecules, were searched for by PCR. The two-LTR PCR primers were positioned at the terminus of the linear genome, allowing amplification across the junction of the LTR only after a covalently closed junction had formed. Also, to ensure that the PCR products detected were the result of newly synthesized viral DNA, 50 µM AZT was included 2 h prior to and throughout the experiment. Aphidicolin was definitely removed from half of the cultures (−Aph) at the time of infection (*T*<sub>0</sub>) but maintained in the other half (+Aph) until completion of the experiment. A final negative control for the PCR consisted of mixing the viral supernatant with mock-infected cells just prior to DNA extraction (lanes m in Fig. 1B and D). Cell cycle analysis showed that at *T*<sub>0</sub>, a large majority of the target cells were, and remained, in the G<sub>1</sub> and S phases except when aphidicolin was removed (Fig. 1A). Total recombinant MLV DNA was monitored with primers that spanned the LTR and *gag* regions. This allowed the detection of RT products that had been largely or fully completed, as well as integrated MLV DNA and one- and two-LTR circles (52). PCR results showed that with the LTRU3 and *gag* primers, the RT was detectable at 2 h postinfection, and more importantly, a two-LTR junction molecule was also seen as early as 1 h postinfection both in the presence and in the absence of aphidicolin (Fig. 1B). The observation that the two-LTR signal was more obvious at 1 h compared to the full-length viral DNA was attributed to a difference in PCR sensitivity. None of the two viral sequences could be amplified in the presence of AZT, indicating that both were neosynthesized early after virus en-

try. To further confirm the efficiency of the drugs, 5-bromo-4-chloro-3-indolylphosphate (X-Gal) staining was done 24 h postinfection. In the presence of either aphidicolin or AZT, none of the MLV-infected NIH 3T3 cells expressed the β-galactosidase transgene whereas target cells grown in the absence of both drugs showed a high level of transduction (corresponding to a titer of 5 × 10<sup>6</sup> to 1 × 10<sup>7</sup> transducing U/ml) (data not shown).

Next we quantified the PCR products in another set of identical experiments (Fig. 1C) by using fluorescence-monitored PCR. These experiments confirmed that the two-LTR junction molecules could be detected as early as 1 h postinfection (but not before; data not shown) in the absence and also in the presence of aphidicolin. Overall, in the absence of aphidicolin, 4.5 × 10<sup>4</sup> ± 0.4 × 10<sup>4</sup> and 6.0 × 10<sup>4</sup> ± 0.9 × 10<sup>4</sup> copies of two-LTR junction molecules per 10<sup>6</sup> cells (*n* = 2) were detected 2 and 4 h, respectively, post recombinant MLV infection (Fig. 1C) whereas under the same experimental conditions, 2.0 × 10<sup>5</sup> ± 0.4 × 10<sup>5</sup> and 1.2 × 10<sup>6</sup> ± 0.02 × 10<sup>6</sup> copies of full-length viral DNA per 10<sup>6</sup> cells were found at the same time points (data not shown). In the presence of aphidicolin, similar results were obtained with 4.0 × 10<sup>4</sup> ± 0.2 × 10<sup>4</sup> and 5.0 × 10<sup>4</sup> ± 0.6 × 10<sup>4</sup> copies of two-LTR junction molecules per 10<sup>6</sup> cells (*n* = 2) detected 2 and 4 h, respectively, after vector entry (Fig. 1C). Simultaneously, we found 1.7 × 10<sup>5</sup> ± 0.2 × 10<sup>5</sup> and 1.40 × 10<sup>6</sup> ± 0.05 × 10<sup>6</sup> copies of full-length viral DNA per 10<sup>6</sup> cells (*n* = 2) (data not shown). Importantly, fewer than 10<sup>3</sup> copies of either two-LTR junction or full-length viral DNA were detected in the presence of AZT.

Altogether, these results showed that with a replication-defective recombinant MLV vector, passage through mitosis was not required for the formation of a neosynthesized two-LTR junction molecule which, indeed, was detected in cells that were predominantly arrested in the G<sub>1</sub> and S phases.

**Two-LTR junction molecules are also detected in replication-competent MLV-infected aphidicolin-arrested cells.** To check that the above observation was not restricted to a replication-defective MLV vector, aphidicolin-arrested NIH 3T3 cells, obtained as described above, were infected with replication-competent MLV for 1, 2, 4, 8, and 24 h. Because the viral supernatant exhibited low infectious titers, the maximum MOI was 0.05 (i.e., 20 times lower than that of the replication-defective vector). Cell cycle analysis of aphidicolin-arrested cells showed that more than 98% of the cells were, and remained, in the G<sub>1</sub> and S phases (data not shown). PCR results showed that full-length and two-LTR junction molecules were seen as early as 2 h postinfection both in the presence and in the absence of aphidicolin (Fig. 1D). Here too, none of these molecules could be amplified when cells were incubated with AZT. Overall, in the absence of aphidicolin, ~2 × 10<sup>2</sup> copies of two-LTR junction molecules per 10<sup>6</sup> cells were detected 2 and 4 h post replication-competent MLV infection (Fig. 1E) whereas under the same experimental conditions, similar results were obtained with ~2 × 10<sup>3</sup> copies of full-length viral DNA per 10<sup>6</sup> cells at the same time points (data not shown). In the presence of aphidicolin, ~10<sup>2</sup> and ~4 × 10<sup>2</sup> copies of two-LTR junction molecules per 10<sup>6</sup> cells were detected at 2 and 4 h, respectively, after virus entry (Fig. 1E). Simultaneously, we found ~2 × 10<sup>3</sup> and ~3 × 10<sup>4</sup> copies of full-length viral DNA per 10<sup>6</sup> cells (data not shown). Again, marginal numbers of copies of either two-LTR junction or full-length

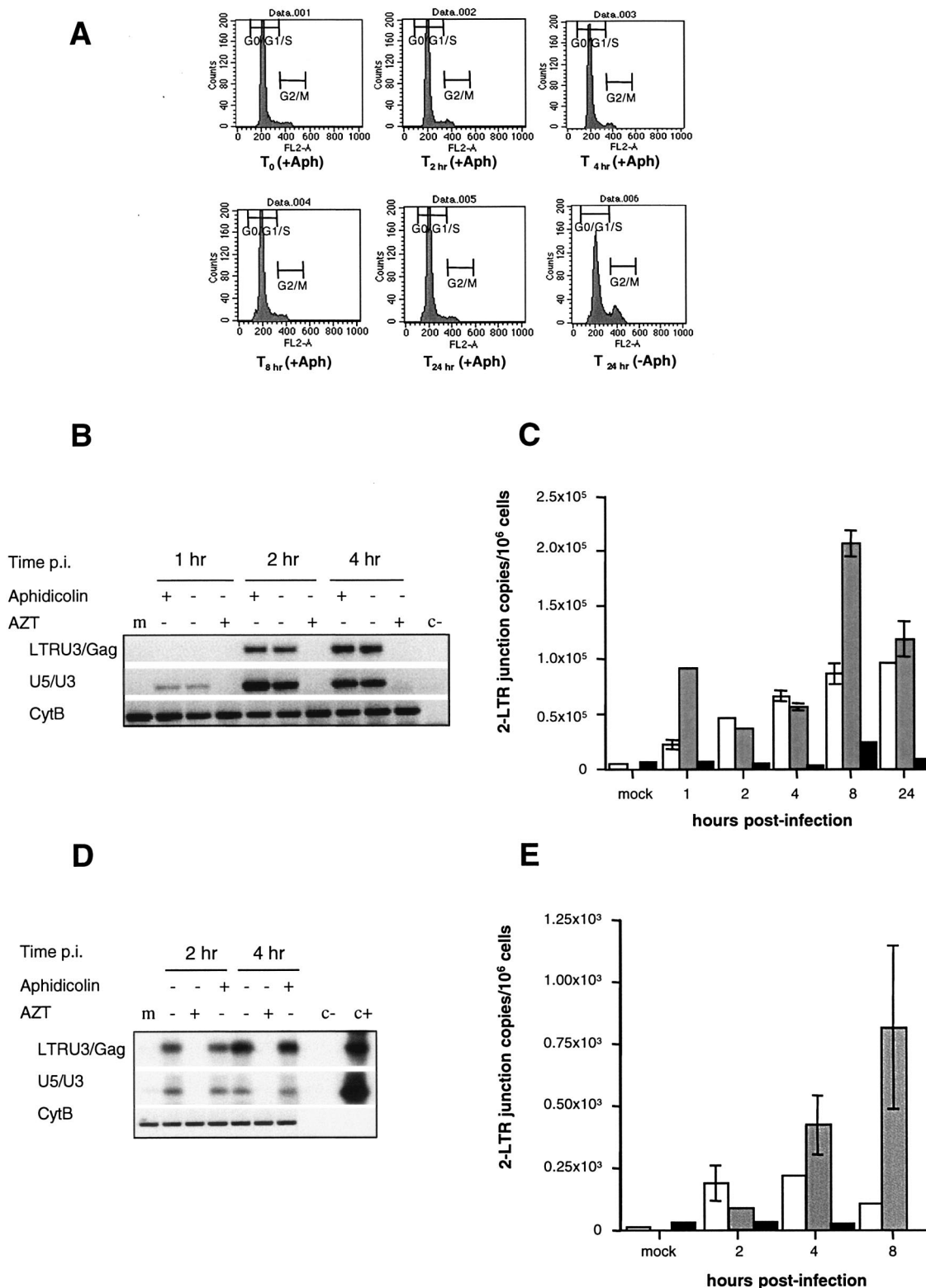


FIG. 1. Viral DNA analysis in aphidicolin-arrested MLV-infected NIH 3T3 cells. (A) Cell cycle analysis of NIH 3T3 cells synchronized and arrested by aphidicolin treatment (+Aph) or not arrested (-Aph) before and after replication-defective MLV infection ( $T_0$  through  $T_{24\text{ hr}}$ ). (B and D) NIH 3T3 cells were infected with replication-defective MLV vectors (B) or replication-competent MLV (D) and kept with (+) or without (-) aphidicolin, in the presence (+) or absence (-) of AZT as described in Materials and Methods. For each time point indicated, cells were recovered and analyzed for their viral DNA contents by PCR, followed by agarose electrophoresis in panel B and Southern blot analysis in panel D, by looking at the detection of the full-length viral DNA (LTRU3/Gag) and the two-LTR junction (U5/U3). (C and E) Total DNA collected at different time points post entry of replication-defective (C) or replication-competent (E) MLV (1 through 24 h), without (open bar) or with (grey bar) aphidicolin or AZT (black bar), was subjected to a quantitative PCR assay for two-LTR junctions, and the results are expressed as the number of copies per million cells. The negative controls (lane c-) consisted of water alone and noninfected cells mixed with the viral supernatant just prior to DNA extraction (lane m [mock infection]). Note that the y axes have different scales. p.i., postinfection.

### Theoretical U5/U3 junction

U5 ← → U3  
 ..accggtcagcgggggtctttcatt atgaaagacccacctgtaggttt...

### Aphidicolin 3T3 arrested-cells

U5 ← → U3  
 1h ..accggtcagcgggggtctttcatt atgaaagacccacctgtaggttt...  
 2h ..accggtcagcgggggtctttcatt tgaaagacccacctgtaggttt...  
 4h ..accggtcagcgggggtctttcatt tgaaagacccacctgtaggttt...  
 8h ..accggtcagcgggggtctttcatt tgaaagacccacctgtaggttt...  
 24h ..accggtcagcgggggtctttcatt tgaaagacccacctgtaggttt...

### Cycling cells

24h (1)..accggtcagcgggggtctttcatt tgaaagacccacctgtaggttt...  
 24h (2)..accggtcagcgggggtctttcatt atgaaagacccacctgtaggttt...  
 tgggggctcgtcgtccgggatg

FIG. 2. Cloning and sequencing of the U5-U3 junction PCR products. Total DNA extracts of infected cells recovered at different times (1 through 24 h) post MLV vector infection and two-LTR junctions were amplified as described in Materials and Methods. The PCR products were cloned and sequenced. Sampled clones are presented along with the theoretical U5-U3 junction and PCR products obtained from cycling cells.

viral DNA were detected in the presence of AZT. Altogether, these data indicated that although the copy numbers were ~100 times lower than in replication-defective virus-infected cells, the quantitative PCR data indicated that 1 to 5% of the neosynthesized viral DNA was two-LTR junction molecules found at early time points in nondividing cells.

**Early two-LTR junction sequences found in aphidicolin-arrested cells are true U5-U3 junctions.** To further characterize our initial observation, the PCR product obtained with the U5 and U3 primers at different time intervals post recombinant MLV infection of aphidicolin-arrested NIH 3T3 cells were cloned into a PCR2.1 plasmid (see Materials and Methods) and sequenced. As expected on the basis of the deduced restriction map from the blunt-ended ligation of the MLV 3' U5 LTR with the 5' U3 LTR, all clones were compatible with the insertion of a true and complete two-LTR junction (data not shown). Several clones were obtained at different times post MLV vector entry and sequenced. A single sequence for each time point is presented in Fig. 2 along with the published junction sequence (class I insert) resulting from blunt-end ligation of the two MLV LTRs (43). All clones exhibited a two-LTR junction, including eight clones obtained at 1, 2, and 4 h post virus entry into aphidicolin-arrested NIH 3T3 cells. When analyzed in detail, most of our clones exhibited an intact 3' end of the U5 sequence with a 2-bp (AA) deletion from the 5' end of the U3 region, except at 1 h post virus entry, when only one A nucleotide was deleted in the single clone isolated. One clone derived by a PCR made on viral DNA and extracted from dividing cells 24 h postinfection (positive controls) showed the same profile. Another had a single A nucleotide

deletion combined with a 22-bp insertion derived from the primer binding site, as described previously in reference 9.

Altogether, these data confirm that the sequence that we amplified by PCR is an authentic U5-U3 junction molecule obtained in aphidicolin-arrested cells as early as 1 h postinfection.

**Other cell lines can support early formation of two-LTR junctions.** We then asked whether the two-LTR viral junction molecule could be formed in two other nonmurine cell lines and whether it would also occur soon (<8 h) after viral entry. Because serum starvation before aphidicolin incubation resulted in dramatic cell apoptosis in cells other than NIH 3T3 cells and since two-LTR junction molecules were detected early in MLV-infected NIH 3T3 cells both in the absence and in the presence of aphidicolin, we used nonarrested TE671 cells derived from a human medulloblastoma (Fig. 3) and ARPE-19 cells from a human retinal epithelial cell line (data not shown). Also, excluding aphidicolin removes its possible interference with the nuclear import-export machinery. The cells were infected with the replication-defective MLV vector in the presence or absence of AZT. Cells were recovered after different periods of incubation (1, 2, 4, 8, and 24 h) postinfection, and total DNA extracts were analyzed by PCR (Fig. 3A) or quantitative PCR for the full-length viral DNA (data not shown) and two-LTR junction molecules (Fig. 3B). PCR results for both cell lines were similar to those obtained with NIH 3T3 cells. Briefly, the full-length DNA and the two-LTR junction molecules were detected as early as 1 to 2 h postinfection and persisted thereafter. The lack of DNA amplification products in the presence of AZT, particularly at early time points, indicated that the viral templates seen were neosynthesized. Quantitative analysis of the two viral DNA molecules confirmed that at 2 and 4 h post MLV infection,  $4.0 \times 10^2$  and  $1.0 \times 10^4$  copies of two-LTR junction molecules (Fig. 3B) and  $1.0 \times 10^4 \pm 0.6 \times 10^4$  and  $5.0 \times 10^5 \pm 0.6 \times 10^5$  copies of full-length DNA templates ( $n = 2$ ) (data not shown) per  $10^6$  TE671 cells, respectively, were formed. The numbers of copies obtained in ARPE-19 cells were in the same range. The percentage of two-LTR molecules versus the full-length product varied from 4% at 2 h to 2% at 24 h postinfection. Low numbers of viral DNA copies of both full-length and two-LTR junction products were detected at 1 h postinfection, which was in agreement with the weak signals found by PCR (Fig. 3A). However, they were considered rather unreliable for an accurate quantitative analysis. Again, the lack of a quantifiable viral template in the presence of AZT confirmed that both products were neosynthesized, reverse-transcribed MLV molecules.

As for the aphidicolin-arrested NIH 3T3 cells, we tested the replication-competent MLV strain on TE671 cells at an MOI of 0.5 and found similar results. Indeed, the full-length viral DNA and the two-LTR junction molecules were detectable as early as 2 h postinfection except when cells were incubated in the presence of AZT (Fig. 3C). Quantitative analysis of the two viral DNA molecules confirmed that at 2 and 4 h post MLV infection,  $\sim 7 \times 10^3$  and  $\sim 1 \times 10^4$  copies of two-LTR junction molecules (Fig. 3D) and  $6 \times 10^5$  and  $6 \times 10^6$  copies of full-length DNA templates (data not shown) per  $10^6$  TE671 cells, respectively, were formed. The percentage of two-LTR molecules versus the full-length product was ~1% at 2 h and 1.5% at 24 h postinfection. In the presence of AZT, a 2-log reduction in copy numbers was consistently found, confirming that

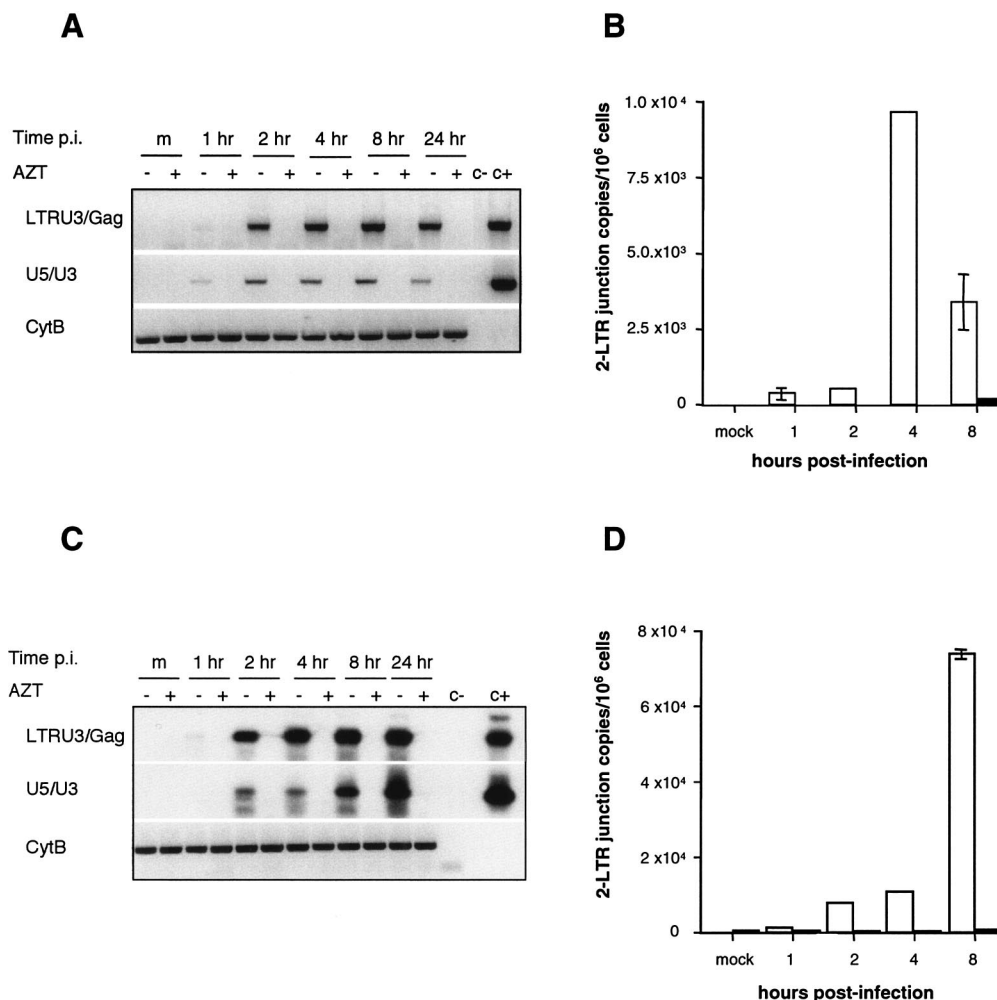


FIG. 3. Viral DNA analysis in nonarrested MLV-infected TE671 cells. (A and C) Human cells were infected with replication-defective (A) or replication-competent (C) MLV for different times (1 through 24 h). Cells were incubated with (+) or without (–) AZT. Total DNA was extracted and subjected to PCR amplification for full-length DNA (LTRU3/Gag), two-LTR junctions (U5/U3), and cytochrome *b* (loading control). Total DNA extracts from noninfected cells mixed with the viral supernatant just prior to DNA extraction (lane m) and water alone (c–) were used as negative controls. The positive controls (c+) were pMFGLZ for the LTRU3/Gag sequence and pPCR2.1U5/U3 for the U5-U3 junction (see Materials and Methods). (B and D) The same extracts were used for quantitative PCR analysis for the two-LTR junctions without (open bar) or with (solid bar) AZT. The negative control consisted of noninfected cells mixed with the viral supernatant just prior to DNA extraction (mock infection). Note that the y axes have different scales. p.i., postinfection.

both products were neosynthesized, reverse-transcribed MLV molecules.

Although the two cell lines were cycling and not aphidicolin arrested, making the origin of the late (8 and 24 h) two-LTR viral products somewhat uncertain, the early (1, 2, and 4 h) neosynthesized two-LTR junction molecule was detectable and quantifiable, with both the replication-competent and replication-defective viruses, suggesting that it may well be generated before nuclear translocation of the linear DNA-containing viral PIC. However, a previous study reported that low levels of viral DNA can be detected by in situ hybridization in the nuclei of Rat-1 cells that remain arrested in aphidicolin (38), a possibility our cell cycle analysis cannot exclude. Therefore, MLV vector-infected TE671 cells were fractionated to look for the cytoplasmic and/or nuclei presence of the early two-LTR junction molecule.

**The early two-LTR junction molecule is found in the cytoplasm of MLV-infected cells.** The formation of MLV two-LTR circles was previously reported to occur late (>12 h) after infection in cells that have been arrested at G<sub>1</sub>/S and released at the time of infection (28, 38). Importantly, in the same studies, the two-LTR circle was described as a valuable index for complete and successful nuclear import of the PIC. However, our data suggested that a neosynthesized two-LTR junction also made of a U5-U3 sequence could be detected and quantified in both aphidicolin-arrested and nonarrested cells as early as 1 to 2 h post virus entry. This apparent contradiction could be fully reconciled if the two-LTR junction molecule that we described in the present study is actually assigned to an early cytoplasmic event preceding the nuclear translocation of the PIC. The assessment of the cellular localization of the early two-LTR junction molecule (cytoplasm versus nucleus) re-

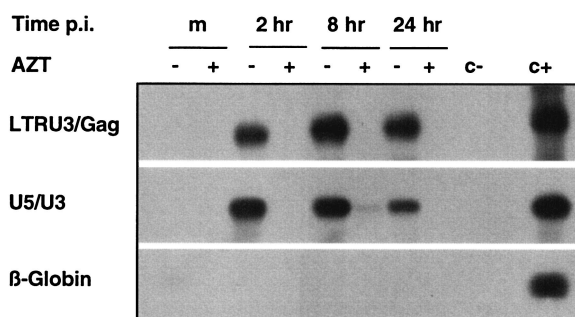


FIG. 4. Cytoplasmic viral DNA forms in TE671-infected cells. Cells were infected with MLV vectors for 2, 8, and 24 h with (+) or without (-) AZT. Cells were recovered, and cytoplasmic and nuclear fractions were obtained as described in Materials and Methods. Cytoplasmic DNA was subjected to PCR amplification, followed by Southern blot analysis for detection of the two-LTR junction sequence. PCR for  $\beta$ -globin, followed by Southern blot analysis, was used to detect possible nuclear contamination. Additional negative controls consisted of cytoplasmic DNA extracts from mock-infected cells (lane m) and water (c-). The positive controls (c+) used were pMFG LZ for the LTRU3/Gag sequence, pPCR2.1U5/U3 for the U5-U3 junction, and total DNA extracts from noninfected cells for the  $\beta$ -globin PCR. p.i., postinfection.

quired a carefully controlled protocol in which each fraction was analyzed by PCR, followed by Southern blot analysis to further enhance the sensitivity for the detection of cross-contaminant DNA. TE671 cells were infected with the recombinant MLV vector and recovered by trypsinization at different times postinfection (2, 8, and 24 h). Cytoplasmic and nuclear extracts were obtained (see Materials and Methods), and a PCR assay for the  $\beta$ -globin DNA sequence was used to test for nuclear DNA contamination in cytoplasmic fractions, whereas the 12S mitochondrial gene was searched for as a cytoplasmic DNA contaminant in nuclear fractions. Despite several attempts, nuclear fractions were reproducibly found to be contaminated with mitochondrial DNA, whereas in most instances, cytoplasmic extracts free of detectable nuclear DNA contaminants could be obtained. To further ensure that nuclear and episomal MLV DNA (i.e., two-LTR circles) were not sucked out of the nuclei during our cell fractionation procedure, leading to false-positive PCR results for the cytoplasmic fraction, we fractionated a G418-grown human kidney carcinoma cell line transfected with a plasmid containing the Epstein-Barr virus *ori*, EBNA, and neomycin resistance genes. The EBNA sequence was searched for by PCR in both cytoplasmic and nuclear fractions, followed by hybridization with an EBNA probe (see Materials and Methods). The amplified EBNA product was only detected in the nuclear fraction (data not shown). Both control tests indicated that our cell fractionation procedure yielded a relevant cytoplasmic extract and also that small nuclear episomal DNA molecules were excluded from it. Therefore, only the controlled cytoplasmic fractions were analyzed for the presence of the two-LTR junction molecule. A Southern blot analysis of amplified products with either the  $\beta$ -globin- or U5-U3-labeled probe is shown Fig. 4. Control samples (lanes a, b, and c) from mock-infected cells, viral supernatant, and water, respectively, as well as the AZT-treated TE671 cells, showed no signal, whereas the two-LTR junction molecule was clearly detectable in the cytoplasm at 2 h post virus entry (in three independent experiments). We also

found that the U5-U3 template was still detectable in the cytoplasmic fraction 24 h postinfection. It is noteworthy, and in agreement with a previous study showing that two-LTR circles from HIV reside in a nucleoprotein complex (7), that our two-LTR junction molecule was detected only if the cytoplasmic fraction was treated with protease prior to viral DNA extraction and amplification (see Materials and Methods).

By using sensitive methods and two independent control tests, we reproducibly demonstrated that an authentic neosynthesized reverse-transcribed MLV U5-U3 junction molecule was present in the cytoplasm early after virus entry.

**The transduction-defective A549-derived virions yield the complete reverse transcriptase product but not the two-LTR junction molecule.** With the chimeric recombinant adenoviruses separately containing MLV *gag-pol*, *env*, and the vector, we previously showed that retroviral vectors produced from HT1080 cells (HT1080-MLV) were able to transduce TE671 target cells efficiently. Virions produced on A549 cells (A549-MLV), on the contrary, were found to be poorly infectious (40). We correlated this discrepancy with the lack of detectable two-LTR circles and proposed, at that time, that the A549-MLV-derived PIC was not competent for nuclear translocation and subsequent integration (40). Combining PCR and Southern blot analyses, we next looked for both the LTRU3-*gag* and U5-U3 junction DNAs at different time intervals in TE671 target cells infected with either HT1080-MLV virions (i.e., MLV vector collected 48 h after transduction of HT1080 cells with the chimeric recombinant adenoviruses coding for the complete MLV particle) (Fig. 5A) or A549-MLV virions (i.e., MLV vector collected 48 h after transduction of A549 cells with the same adenoviruses) (Fig. 5B). AZT was included as a control for the neosynthesized products. The presence of a residual AZT-resistant band in Fig. 5B, 8-h lanes, was found when the chimeric recombinant adenoviruses encoding the MLV vector that occasionally contaminates retroviral supernatants was used. However, the important observation was that, as early as 2 h postinfection, HT1080-MLV yielded late reverse-transcribed products, as well as the two-LTR junction DNA, whereas only the former was detectable post A549-MLV entry. Since the lack of a two-LTR junction correlated with the lack of transduction despite the presence of detectable LTRU3-*gag* products, it suggested that the early U5-U3 junction DNA is possibly required for efficient transduction.

## DISCUSSION

Retroviral linear DNA is the precursor to the integrated provirus (3, 5, 17), while inside the nucleus two other forms of retroviral DNA are traditionally described: covalently closed circular forms with one LTR (the product of recombination between the two LTRs of the linear DNA) or two tandem LTRs resulting either from blunt-end ligation of the duplex linear DNA, producing a circle junction often with CATT-AATG (43, 44) between the LTR termini, or from autointegration, which produces significantly rearranged molecules. These circular DNA molecules were initially described in nuclei of avian sarcoma virus-infected cells (19, 41-43, 49). These closed circular forms that are formed upon nuclear entry of the linear DNA molecule are unnecessary for integration, although a recent study suggested that under some circum-

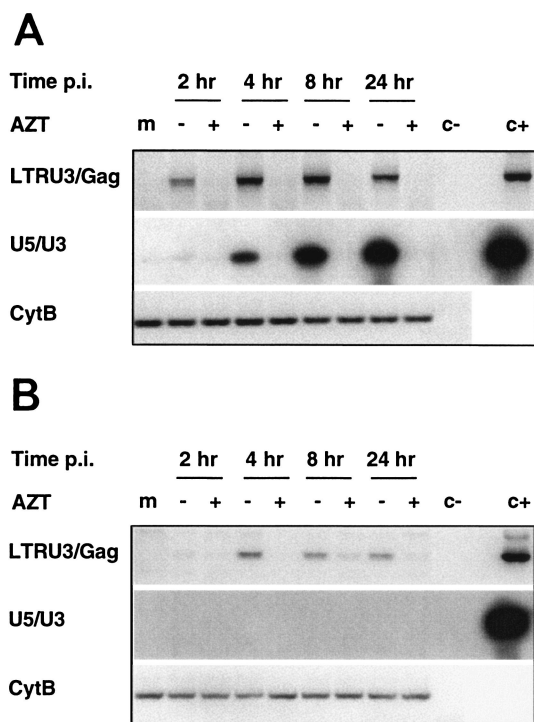


FIG. 5. Viral DNA analysis of HT1080-MLV- and A549-MLV-infected TE671 cells. Cells were infected with HT1080-MLV (A) and A549-MLV (B) for 2, 4, 8, and 24 h in the presence (+) or absence (-) of AZT. Cells were recovered, and their viral DNA contents were analyzed by PCR and Southern blot analyses for the detection of full-length viral DNA (LTRU3/Gag) and the two-LTR junction (U5/U3). Mock-infected cells (m) and water alone (c-) were used as negative controls. The positive controls (c+) used were pMFLZ for the LTRU3/Gag sequence and pPCR2.1U5/U3 for the U5-U3 junction. CytB corresponds to the cytochrome *b* sequence. p.i., postinfection.

stances the viral IN may use the two-LTR circle junction as a template for integration into the host genome particularly if it is the only, or predominant, species delivered into the nucleus (34). Circular DNA forms are considered to be useful indicators of viral DNA entry into the nucleus (4) but as dead-end products (nonproductive pathway) (5, 17, 30). Similar molecules are found in the nuclei of HIV-infected cells, both in vitro and in vivo (15, 24), but this observation was recently challenged by the hypothesis that the use of LTR circle formation as a marker of virus-mediated nuclear import may be less definitive than was previously thought (14). Recently, the episomal two-LTR circles formed during a single round of HIV-1 replication were found to persist indefinitely in a transformed CD4<sup>+</sup> T-cell line (35) and in SupT1 lymphoid cells (8), decreasing in concentration only as a function of dilution resulting from cell division.

Mitosis has previously been established as the essential cell cycle component needed for oncoretroviral infection (28, 38). The importance of mitosis in the life cycle of MLV is commonly attributed to the breakdown of the nuclear membrane allowing the large PIC containing the linear viral DNA to reach the host genome for subsequent integration. Therefore, circular MLV DNA could only be expected at the time of mitosis (28).

Herein, we report the amplification of a two-LTR DNA

sequence as early as 1 to 2 h after entry of a recombinant MLV vector. Significantly, we made the same observation with a replication-competent MLV, suggesting that this phenomenon may be indicative of a natural process in the early step of MLV cell entry. This finding was surprising because it meant that either a significant fraction of MLV vector had reached the nucleus that quickly or two-LTR junction molecules (not necessarily two-LTR circles) were generated in the cytoplasm soon after MLV entry. When NIH 3T3 cells were synchronized and then treated with aphidicolin for 18 h, serial DNA content analyses by flow cytometry showed primarily a G<sub>1</sub> peak, with a small number of cells arrested in mid-S phase and no cells arrested in G<sub>2</sub>. Despite this nonproliferating status, the two-LTR junction sequence could still be amplified, suggesting that this molecule was indeed synthesized independently of mitosis. We made the same observation on primary unstimulated human T lymphocytes transduced with a gibbon ape leukemia virus-pseudotyped MLV encoding for GFP (Serhan et al., unpublished data). Despite the technical difficulties associated with cell fractionation, but with the availability of appropriate and relevant control assays, the two-LTR junction molecules were found in the cytoplasm as early as 2 h postinfection. Because partial reverse transcripts are found in MLV virions (47), AZT was included in control experiments. Also, extensive DNase treatment was applied to our retroviral supernatants because of possible two-LTR circle molecule contamination from producer cell death. Altogether, these data indicated that a neosynthesized authentic two-LTR junction molecule was generated in the cytoplasm as early as 2 h post MLV entry. A possible related finding is in a recent study in which the kinetics of viral DNA synthesis was studied in BHK-21 cells infected with a human foamy retrovirus and two-LTR circles were initially detected in the cytoplasmic fraction 3 h postinfection (11). Whether the molecule that we have described is a circle or a concatemered duplex linear DNA remains unknown.

Why did we detect a two-LTR junction sequence in the cytoplasm while previous studies restricted it to the nucleus? First, we looked at early time points, starting as early as 1 h post vector entry; second, we used total DNA and combined a sensitive PCR assay with Southern analysis, which were rarely used conjointly, at the time two-LTR circle molecules were attributed to the nuclei of retrovirus-infected cells. Also, most studies analyzed Hirt supernatant-derived DNA, starting 8 h postinfection at the earliest. However, one study analyzing the requirement for mitosis in the MLV life cycle used total DNA extract and looked for the presence of two-LTR circles as early as 2 h postentry of an MLV-based replication-defective vector (28). In addition, they searched for the two-LTR sequence resulting from end-to-end ligation by PCR and subsequently used an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled probe specific to the U5-U3 junction. Finally, their material was obtained from synchronized E36 cells, a Chinese hamster lung cell line that had been released from hydroxyurea (HU) treatment and allowed to proceed through the cell cycle. In this case, the two-LTR circular form was not detected until 8 h after infection, when the majority of cells have passed through mitosis. Because HU—and not aphidicolin—inhibits deoxynucleotide synthesis and consequently blocks HIV-1 DNA synthesis by decreasing the amount of intracellular deoxynucleotides (31), it is a reasonable explanation for the lack of two-LTR detection in this

study at early time points. Indeed, it may require 8 h post HU release to allow efficient DNA synthesis with the generation of detectable two-LTR circle molecules. In support of this hypothesis is the fact that in vitro direct ligation of the ends of HIV-1 linear DNA molecule with cytoplasmic extracts is induced by incubation of the extracts in the presence of added deoxynucleoside triphosphates. To the contrary, no two-LTR junctions were detected when extracts were incubated in the absence of added deoxynucleoside triphosphates (15). In another study, circular forms of viral DNA were specifically unsuccessfully searched for in cytoplasmic extracts of replication-competent MLV-infected cells by a direct Southern blot assay, the sensitivity of which was calibrated with cloned MLV DNA and found to detect as few as  $4 \times 10^5$  circular DNA molecules (4). In our study, the quantitative PCR, because of its exquisite sensitivity, was able to detect as few as  $\sim 10^2$  two-LTR junction molecules in NIH 3T3-infected cells, providing an additional explanation for our ability to detect this sequence in cytoplasmic extracts within 2 h postinfection.

In all three cell types, NIH 3T3, TE671, and ARPE-19, infected with a replication-defective MLV vector, if the 1-h time point quantification was rather unreliable owing to the low signal levels and the large excess of chromosomal DNA relative to episomal nonintegrated MLV DNA (46), the number of full-length DNA template copies was reproducibly  $\sim 20$  times higher than the copy number of two-LTR junction molecules at 2 and 4 h postinfection (but 5 times higher at the 2-h time point in NIH 3T3 cells). This difference increased up to  $\sim 50$  times at later time points (24 h). If one assumes that the quantitative PCR assay displayed similar sensitivity, then this suggests a possible temporal relationship between the completion of RT and the emergence of the two-LTR junction sequence. Although partial reverse transcripts from incoming virions (47) could conceivably artificially produce such a sequence, the effect of AZT indicated that the RT step occurring in the cytoplasm was required for the two-LTR molecule to emerge. The facts that, on the one hand, introduction of AZT resulted in complete absence of the two-LTR junction sequence and that, on the other hand, a reverse transcriptase inhibitor such as AZT is considerably more active on full-length viral DNA than on short reversed templates, i.e., minus strand strong-stop DNA (36), are consistent with the scenario in which the two-LTR junction sequence that we detected at early time points in the cytoplasm arose from complete reverse transcripts rather than from earlier and shorter intermediates. This is also consistent with the fact that A549-MLV vectors, as opposed to HT1080-MLV vectors, can generate late RT products but not the two-LTR junction. The hypothesis that the complete linear viral DNA is the likely substrate for the two-LTR junction molecule that we detected early in the cytoplasm is also in agreement with the observation that linear HIV-1 DNA present in cytoplasmic extracts can circularize in vitro to form one- and two-LTR circles in the presence of added nucleoside triphosphates (15).

The two-LTR molecule that we detected in the cytoplasmic fraction is suspected to be associated with a protein(s) because proteinase K treatment prior to PCR was required to amplify the U5-U3 junction sequence. Untreated samples were reproducibly found to be negative. The use of digitonin, a mild detergent, in the fractionation procedure may account for this

requirement. However, the associated protein(s) remains to be identified and among the possible candidates, the viral IN is a central one. Indeed, if the early cytoplasmic two-LTR junction molecule that we have described here is part of the productive infectious pathway, which is suggested by the fact that the noninfectious A549-MLV strain did not generate two-LTR junction molecules, then the presence of the viral IN would be expected. On the contrary, if this molecule belongs to the abortive pathway, then it may be associated not with the viral IN but rather with a nucleoprotein complex different from the PIC, as shown previously for HIV-1 two-LTR circles (7). If our sequencing data obtained from only one to three two-LTR PCR2.1-derived clones per time point were critical to confirm the specificity of our PCR, they remain poorly informative with respect to the possible processing of the LTR ends by the viral IN, resulting, for instance, in autointegration (15, 22). A comprehensive cloning-and-sequencing analysis of the U5-U3 junction obtained at different time points after virus entry and studies aimed at identifying the overall structure (i.e., circles and/or concatemerized duplex linear DNA) are currently under way to further characterize this early cytoplasmic molecule in the MLV life cycle.

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